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PCT/KR2004/000680

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Artcle 36 and Rule 70)

Applicant's or agent's file reference 4FPO-03-06	FOR FURTHER ACTION	See Form PCT/IPEA/416
International application No. **PCT/KR2004/000680**	International filing date *(day/month/year)* **25 MARCH 2004 (25.03.2004)**	Priority date *(day/month/year)* 25 MARCH 2003 (25.03.2003)

International Patent Classification (IPC) or national classification and IPC

IPC7 C12N 9/14, C12N 15/52, A23K 1/165

Applicant

Republic of National Fisheries Research and Development Institute et al

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ (sent to the applicant and to the International Bureau) a total of 10 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) _____, containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box relating to Sequence Listing (see Section 802 of the Administrative Instructions).
4. This report contains indications relating to the following items:
   - ☒ Box No. I Basis of the report
   - ☐ Box No. II Priority
   - ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - ☐ Box No. IV Lack of unity of invention
   - ☒ Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - ☐ Box No. VI Certain documents cited
   - ☐ Box No. VII Certain defects in the international application
   - ☐ Box No. VIII Certain observations on the international application

Date of submission of the demamd	Date of completion of this report
17 SEPTEMBER 2004 (17.09.2004)	16 AUGUST 2005 (16.08.2005)

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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/KR2004/000680

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☒ This report is based on translations from the original language into the following language English, which is the language of a translation furnished for the purposes of:

   ☐ international search (under Rules 12.3 and 23.1(b))
   ☒ publication of the international application (under Rule 12.4)
   ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this reort as "originally filed" and are not annexed to this report):*

   ☐ the international application as originally filed/furnished

   ☒ the description:
   pages 2 3 9-36 as originally filed/furnished
   pages* 1, 4-8, 39 received by this Authority on 25/04/2005
   pages* _____ received by this Authority on _____

   ☒ the claims:
   pages _____ as originally filed/furnished
   pages* _____ as amended (together with any statment) under Article 19
   pages* 37, 38, 38/1 received by this Authority on 25/04/2005
   pages* _____ received by this Authority on _____

   ☒ the drawings:
   pages 1/5-5/5 as originally filed/furnished
   pages* _____ received by this Authority on _____
   pages* _____ received by this Authority on _____

   ☒ the sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing.

3. ☒ The amendments have resulted in the cancellation of:

   ☐ the description, pages _____
   ☒ the claims, Nos. 4
   ☐ the drawings, sheets _____
   ☐ the sequence listing *(specify)*: _____
   ☐ any table(s) related to sequence listing *(specify)*: _____

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

   ☐ the description, pages _____
   ☐ the claims, Nos. _____
   ☐ the drawings, sheets _____
   ☐ the sequence listing *(specify)*: _____
   ☐ any table(s) related to sequence listing *(specify)*: _____

** If item 4 applies, some or all of those sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
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Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	None	YES
	Claims	1-3, 5-12	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-3, 5-7	NO
Industrial applicability (IA)	Claims	1-3, 5-12	YES
	Claims	None	NO

2. Citations and explanations (Rule 70.7)

The following documents have been considered for the purpose of this report:

D1: Appl. Microbiol. Biotechnol., Vol. 57, pp. 474-481 (2001).
D4: 9th International Symposium on the Genetics of Industrial Microorganisms, Abstract Book P21-31, pp. 222 (JULY 2002)
[KIM Y.O. et al., 'Purification and characterization of a novel phytase from *Citrobacter braakii* YH-15'].

D1 discloses microbial phytases developed by genetic engineering based on the gene sequences and protein structures; characteristics of different heterologous phytase expression systems, including those of plants, bacteria, fungi, and yeast; and the use of said phytase as a feed additive.

D4 discloses a novel phytase with the specific activity of 3,457 U/mg, the molecular weight of 47 kDa, the optimum pH of 4.0, the optimum temperature of 50 °C and the Km of 0.46 mM, isolated from *Citrobacter braakii* YH-15; and the use of said phytase as a feedstuff to nonruminants.

I. Novelty & Inventive Step

1) Claims 1-3 and 5-7

Claims 1-3 and 5-7 relate to an isolated protein comprising an amino acid sequence of SEQ ID NO.2 at its N-terminus and having (a) molecular weight of 47 kDa, (b) optimal pH of 3.5-4.5, (c) optimal temperature of 45-55 °C, (d) phytase as a substrate, (e) Michaelis constant of 0.3-0.5 mM, (f) high resistance to protease, and (g) specific activity at least 1,500 U/mg; and a gene encoding the said protein. D4 discloses a phytase isolated from *Citrobacter braakii* YH-15, sharing the identical characteristics with the protein of the present invention.

(Continued on Supplemental Sheet.)

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Supplemental Box Relating to Sequence Listing

Continuation of Box No. I, item 2:

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this report was established on the basis of:

   a. type of material
      - [x] a sequence listing
      - [ ] table(s) related to the sequence listing

   b. format of material
      - [x] in written format
      - [x] in computer readable form

   c. time of filing/furnishing
      - [x] contained in the international application as filed
      - [x] filed together with the international application in computer readable form
      - [ ] furnished subsequently to this Authority for the purposes of search and/or examination
      - [ ] received by this Authority as an amendment* on _____

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed of furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

Box No. V

Therefore, claims 1-3 and 5-7 in this invention are not considered to be novel under PCT Article 33(2).

The technical feature of this invention is to determine the N-terminal and full amino acid sequence of the phytase known in the prior art document D4 and the gene encoding said phytase. Because D1 discloses microbial phytases developed by genetic engineering based on the gene sequences and protein structures, and the characteristics of different heterologous phytase expression systems, it is obvious to a person skilled in the art to determine the N-terminal and full amino acid sequence of the protein known in the prior art document.

Therefore, the subject matter of claims 1-3 and 5-7 does not appear to involve an inventive step under PCT Article 33(3).

2) Claims 8-12

Claims 8-12 relate to a microorganism belonging to *Citrobacter* sp., *Citrobacter braakii* and strain YH-15 (KCCM 10427) producing said phytase; and a feed additive containing said phytase or said microorganism.

D4 discloses *Citrobacter braakii* YH-15 strain producing said phytase, identical to the microorganism of this invention; and the use of said phytase or said microorganism as a food and feed additive.

Therefore, claims 8-12 in this invention are not considered to be novel under PCT Article 33(2).

II. Industrial Applicability

The subject matter of claims 1-3 and 5-12 is considered to be industrially applicable under Article 33(4). //

PHYTASE PRODUCED FROM CITROBACTER BRAAKII

FIELD OF THE INVENTION

The present invention relates to a novel phytase enzyme, a gene coding the enzyme, a *Citrobacter* species producing the enzyme and a feed additive containing the protein or the strain as an effective ingredient.

BACKGROUND

Phytase is an enzyme decomposing phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate) to produce phosphate and phosphate inositol. Phytic acid takes 50~70% of phosphorus contained in animal feed grains. However, monogastric animals such as fish, fowls and pigs do not have phytase decomposing phytic acid inside body, so that a coefficient of utilization of vegetable phosphorus, which is necessary for growth, is very low, requiring an enough supply from outside body in the form of inorganic compounds. Phytic acid included in feed grains, which is not digested in monogastric animals, can be decomposed enzymatically by microorganisms in

plants all over the country and identified thereof. The present inventors completed this invention by confirming that phytase produced by the above microorganism of the invention was a novel protein having a novel base sequence and an excellent titer.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a novel protein decomposing phytic acid produced from a *Citrobacter* species strain and a gene coding the protein.

It is also an object of this invention to provide a *Citrobacter braakii* strain producing the above protein.

It is a further object of this invention to provide a feed additive containing the above protein or the above strain as an effective ingredient.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In order to achieve the above object, the present invention provides a protein produced from

a *Citrobacter* species Strain and having physicochemical characteristics as follows.

(a) Molecular weight : about 47 kDa on SDS-PAGE,

(b) Optimal pH : pH 3.5 - pH 4.5,

(c) Optimal temperature : 45℃ - 55℃,

(d) Substrate specificity : phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP,

(e) Michaelis constant of 0.3 - 0.5 mM utilizing phytate as a substrate,

(f) High resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin.

The present invention also provides a gene coding the above protein.

The present invention also provides a *Citrobacter braakii* strain producing the above protein.

The present invention further provides a feed additive containing the above protein or the above strain as an effective ingredient.

Hereinafter, the present invention is described in detail.

The present invention provides a novel

protein decomposing phytic acid produced from a *Citrobacter* species strain.

The protein having an activity of decomposing phytic acid was named "phytase".

The phytase of the present invention is characterized by having the physicochemical characteristics as follows.

(a) Molecular weight : about 47 kDa on SDS-PAGE,

(b) Optimal pH : pH 3.5 - pH 4.5,

(c) Optimal temperature : 45℃ - 55℃,

(d) Substrate specificity : phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP,

(e) Michaelis constant of 0.3 - 0.5 mM utilizing phytate as a substrate,

(f) High resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin.

Phytase of the present invention is an enzyme having phytase activity, which is originated from *Citrobacter* species strain and can be separated and purified after culturing the strain by using ammonium sulfate precipitation, phenyl separose, DEAE-separose, CM-separose and Mono S HR 5/5

column.

The phytase has a molecular weight of 47 kDa on SDS-PAGE and is activated by using phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP as a substrate. The phytase is an acidic enzyme showing a high enzyme activity at 45℃-55℃ (optimal activity is observed at 50℃). The enzyme activity is very stable between pH 3.0 and pH 7.0, the best activity can be seen between pH 3.5 and pH 4.5, and the optimal pH is 4.0. The enzyme activity is strongly inhibited by Fe^{3+}, Zn^{2+} and Cu^{2+} of various metal ions. Km value to phytate is 0.46 mM, and Vmax value is 6,027 U/mg. Besides, the phytase shows a strong resistance against many proteases such as pepsin, trypsin, papain, elastase or pancreatin (see FIG. 4, Table 5 and Table 6).

The phytase of the present invention is produced from *Citrobacter* species strain, and is preferably produced from *Citrobacter braakii*. More particularly, it is more preferable for the phytase of the present invention to be produced from *Citrobacter braakii* YH-15 (Accession No: KCCM 10427).

The phytase has an amino acid sequence represented by SEQ. ID. No 2 or a N-terminal amino acid sequence containing a sequence represented by SEQ. ID. No 2 in which one or more amino acids are replaced, deleted or added. The amino acid sequence is quite different from that of conventional phytase enzyme, so that it has been confirmed that the phytase of the present invention is a novel enzyme.

It is more preferable for the phytase of the present invention to include not only a N-terminal amino acid sequence represented by SEQ. ID. No 2 but also an amino acid sequence represented by SEQ. ID. No 7 or to have at least 70% homology with the sequences.

It is also preferred for the phytase of the present invention to have at least 1,500 U/mg of specific activity to phytate and is more preferred to have at least 3,000 U/mg of specific activity.

The present invention also provides a gene coding the above protein.

It is preferable for the gene to code an amino acid sequence represented by SEQ. ID. No 7

What is claimed is

1. An isolated protein comprising an amino acid sequence of Seq ID No.2 at its N-terminus wherein said protein having the following characteristics

(a) Molecular weight : about 47 kDa on SDS-PAGE,

(b) Optimal pH : pH 3.5 - pH 4.5,

(c) Optimal temperature : 45℃ - 55℃,

(d) Substrate specificity: phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP,

(e) Michaelis constant of 0.3 - 0.5 mM utilizing phytate as a substrate,

(f) High resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin,

(g) Specific activity to phytate : at least 1,500 units/mg.

2. The protein as set forth in claim 1, wherein the protein comprises an amino acid sequence represented by 23-433 amino acids of Seq ID No.7 or amino acid sequence having over 70% sequence homology with the same.

3. The protein as set forth in claim 1, wherein

the protein comprises an amino acid sequence represented by SEQ ID. No 7 or an amino acid sequence having over 70% sequence homology with the same.

5. The protein as set forth in any one of claims 1 to 3, wherein the specific activity of the protein to phytate is at least 3,000 units/mg.

6. A gene encoding the protein of any one of claims 1 to 3 and 5.

7. The gene as set forth in claim 6, wherein the gene has a base sequence represented by SEQ. ID. No 6 or a base sequence having over 70% sequence homology with the same.

8. A microorganism belonging to *Citrobacter* species producing the protein of any one of claims 1 to 3 and 5.

9. A feed additive containing the protein of any one of claims 1 to 3 and 5, or combination thereof as an effective ingredient.

10. The microorganism as set forth in claim 8, wherein *Citrobacter* species is *Citrobacter braakii*.

11. The microorganism as set forth in claim 10, wherein *Citrobacter braakii* is *Citrobacter braakii* YH-15 strain (Accession No: KCCM

10427).

12. A feed additive containing the microorganism of any one of claims 8, 10 and 11, or combination thereof as an effective ingredient.

ABSTRACT OF THE DISCLOSURE

The present invention relates to a novel phytase enzyme, a gene coding the enzyme, and a *Citrobacter* species producing the enzyme. Particularly, the present invention relates to the phytase enzyme produced from *Citrobacter* sp. having (a) molecular weight of 47 kDa, (b) optimal pH of 3.5-4.5, (c) optimal temperature of 45-55℃, (d) as substrates phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP, (e) Michaelis constant of 0.3-0.5 mM utilizing phytate as substrate, and (f) high resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin. The present invention also relates to the gene coding the phytase enzyme and the *Citrobacter braakii* producing the enzyme. The phytase enzyme and the *Citrobacter braakii* producing the enzyme of the present invention can be used in manufacturing a feed of monogastrics as feed additive and in recovering a specific decomposition product of phytate at low price.